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Patentanmeldung Nr.   **Patent application No.**   **Demande de brevet n°**

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A gene delivery vehicle expressing the apoptosis-inducing proteins VP2 and/or Apoptin

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Title: A gene delivery vehicle expressing the
         apoptosis-inducing proteins VP2 and/or apoptin

The invention relates to gene delivery vehicles which
comprise nucleic acid molecules encoding apoptosis-inducing
proteins VP2 and/or apoptin (VP3) like activity.

Also, the invention relates to anti-tumor therapies
5   and to the diagnosis of cancer. Infection of various human
tumor cells with the gene delivery vehicles of the
invention will result in the induction of apoptosis in
tumor cells and much reduced apoptosis, if at all, in
normal diploid, non-transformed/non-malignant cells.

10

In vitro, expression of the chicken anemia virus
(CAV)-derived protein apoptin (VP3) in chicken transformed
cells induced apoptosis (Noteborn et al. 1994, Noteborn and
Koch, 1995). Apoptosis is characterized by shrinkage of
15 cells, segmentation of the nucleus, condensation and
cleavage of DNA into domain-sized fragments, in most cells
followed by internucleosomal degradation. Finally, the
apoptotic cells fragment into membrane-enclosed apoptotic
bodies, which are rapidly phagocytosed by neighbouring
20 cells. Therefore, apoptosis causes much less destruction of
tissue than necrosis, the non-physiological type of cell
death (Wyllie et al., 1980, Arends and Wyllie, 1991 and
White, 1996).

Apoptin is a small protein, only 121 amino acids long,
25 which is rather basic, and proline-, serine- and
threonine-rich (Noteborn et al. 1991). In the analysed
transformed chicken cells, and which all undergo
apoptin-induced apoptosis, apoptin is located strictly
within the cell nucleus. Truncation of the C-terminal basic
30 stretch of apoptin results in a reduced nuclear location
and a significantly reduced apoptotic activity (Noteborn et
al., 1994).

Apoptin, and other proteins with apoptin-like
activity, can also induce apoptosis in human malignant and

transformed cell lines, but not in untransformed human cell lines. We have established that apoptin-induced apoptosis occurs in the absence of functional p53 (Zhuang et al., 1995a), and cannot be blocked by Bcl-2, BCR-ABL (Zhuang et

5 al., 1995), the Bcl-2-associating protein BAG-1 and the cow-pox protein CrmA (Noteborn, 1996). In vitro, apoptin fails to induce programmed cell death in normal lymphoid, dermal, epidermal, endothelial and smooth-muscle cells. However, when normal cells are transformed they become

10 susceptible to apoptosis by apoptin or other proteins with apoptin-like activity. Long-term expression of apoptin in normal human fibroblasts revealed that apoptin has no toxic or transforming activity in these cells. In normal cells, apoptin was found predominantly in the cytoplasm, whereas

15 in transformed or malignant cells i.e. characterised by hyperplasia, metaplasia or dysplasia, it was located in the nucleus, suggesting that the localization of apoptin is related to its activity (Danen-Van Oorschot et al., 1997, Noteborn, 1996).

20 Apoptosis is an active and programmed physiological process for eliminating superfluous, altered or malignant cells (Earnshaw, 1995). The apoptotic process can be initiated by a variety of regulatory stimuli (Wyllie, 1995 and White, 1996). Changes in the cell survival rate play an

25 important role in human pathogenesis, e.g. in cancer development, which is caused by enhanced cell proliferation but also by decreased cell death (Kerr et al., 1994). A variety of chemotherapeutic compounds and radiation have been demonstrated to induce apoptosis in tumor cells, in

30 many instances via wild-type p53 (Thompson, 1995, Bellamy et al., 1995, Steller, 1995).

Many tumors, however, acquire a mutation in p53 during their development, often correlating with poor response to cancer therapy (Hooper, 1994). For several (leukemic)

35 tumors, a high expression level of the proto-oncogene Bcl-2 is associated with a strong resistance to various

apoptosis-inducing chemotherapeutic agents (Hockenberry, 1994, Kerr et al., 1994, and Sachs and Lotem, 1993).

Therefore, apoptin may become a potential candidate for the destruction of tumor cells, or other cells

5   characterised by hyperplasia, metaplasia or dysplasia, which have become resistant to (chemo)therapeutic induction of apoptosis, due to the lack of functional p53 and (over)-expression of Bcl-2 and other apoptosis-inhiting agents. The fact that apoptin does not induce apoptosis in

10 normal non-transformed human cells, at least not *in vitro*, suggests that a toxic effect of apoptin treatment *in vivo* might be very low.

However, thus far, expression of apoptin in tumor cells is carried out by using transient transfection of

15 tissue-culture cells. The disâdvantage of this expresssion method is the very low percentage of cells, which can express apoptin under *in vitro* circumstances. *In vivo*, the used transfection methods will be cumbersome and not efficient, if possible at all, and will not at all

20 contribute to effective cancer treatment.

Adenovirus can be derived from human adenoviruses (Ads), which are non-enveloped, icosahedral DNA viruses. The genome consists of a linear, double-stranded DNA

25 molecule of about 36 kb carrying inverted terminal repetitions (Horvitz, 1990). The serotypes that have been used for vector development (Ad2 and Ad5) are not associated with severe human pathology (Horvitz, 1990). The virus is extremely efficient in introducing its DNA into

30 the host cell. Ads can infect a wide variety of dividing and non-dividing cells of a broad range of species, and the virus can be produced in large quantities with relative ease. In contrast to retroviruses, Ads do not integrate into the host cell genome. All currently used rAdVs have a

35 deletion in the E1 region, where novel DNA can be introduced. The E1 deletion renders the recombinant virus replication-defective (Stratford-Perricaudet and

Perricaudet, 1991). On the one hand, this provides an essential safety feature: the rAdV cannot replicate on human cells in the absence of E1A proteins. Thus, the rAdV can deliver its genetic information in a human cell, but
5   this will not result in a lytic or productive infection. On the other hand, it poses a problem for the production of these vectors. However, the E1 functions need not necessarily to be encoded by the vector itself. They can also be provided in trans, in special helper cells, which
10   express the E1 genes. Upon infection or transfection of these helper cells with an E1-deleted Ad vector, the cellular E1 proteins will complement the replication of the rAdV, which results in the production of progeny rAdVs. Ad helper cells must be of human origin, and they must contain
15   and express the AdE1 region, i.e. Ad-transformed human cells such as cell line 293 (Graham and Prevec, 1991), the 911 cell line (Fallaux et al., 1996) and the PER.C6 cell line (Fallaux, 1996).

20   The invention now provides a gene delivery vehicle (or vector) which enables using the features of the anti-tumor agent apoptin, or other proteins with apoptin-like activity, for cancer treatment via the use of gene-therapy, or for the treatment of malignancies characterized by
25   hyperplasia, metaplasia or dysplasia. Such a gene delivery vehicle, which is a independently infectious vector can for example be a virus, or a liposome, or a polymer, or the like, that in it self can infect or in any other way deliver genetic information to for example tumor-cells that
30   can be treated. The genetic information comprises a nucleic acid molecule encoding apoptin-like activity. The invention also provides a gene delivery vehicle that greatly has been increased in its capacity to express apoptin-like activity. Surprisingly, it was found that changing upstream non-
35   coding nucleic acid sequences, located within the translation initiation site, that precede apoptin-like protein coding sequences greatly enhances expression of

said protein in tumor cells. The invention also provides a
gene delivery vehicle comprising a nucleic acid encoding a
VP2-like activity. VP2-like activity, surprisingly, was
shown to act synergistically with apoptin-like activity

5   concerning the induction of apoptosis in tumor cells, VP2-
like protein in it self can also act synergistically or
additive to for example (chemo)therapeutic induction of
apoptosis. The invention also provides a gene delivery
vehicle comprising a nucleic acid encoding a VP2-like

10   activity additionally to comprising a nucleic acid molecule
encoding apoptin-like activity. Provided by the invention
is for example a gene delivery vehicle acording to the
invention that is a virus. Additionally, the invention
provides a gene delivery vehicle that in it self is

15   replication-defective virus but which can replicate in
helper or packaging cells to generate progeny gene delivery
vehicles. The gene delivery vehicle thus provided by the
invention can for instance be an adenovirus, or an
retrovirus or other DNA or RNA recombinant viruses that can

20. be used as delivery vehicle or a plasmovirus.
Additionally, the invention provides a gene delivery
vehicle which has additionally been supplemented with a
specific ligand or target molecule or target molecules, by
which the gene delivery vehicle can be specifically

25   directed to deliver its genetic information at a target
cell of choice. Such a target molecule can for instance be
a viral spike protein, or receptor molecule, or antibody,
reactive with a tumor cell surface receptor or protein.

30   Also, the invention provides a gene delivery vehicle which
can be used in the diagnosis i.e. of cancer. Such a gene
delivery vehicle can i.e. be used for in vitro diagnosis,
wherein tissue or cell samples or biopsies are taken from a
human or animal. Such samples can then be evaluated or

35   tested by infecting them, in culture or directly, with said
gene delivery vehicle capable of expressing i.e. apoptin-
like activity. Only transformed cells, or cells displaying

various stages of hyperplasia, dysplasia or metaplasia, or tumor or cancer cells, express protein with apoptin-like activity within the nucleus. The presence of said protein can i.e. be demonstrated with classical (immuno)

5 histochemical techniques i.e. microscopically or with automated cell sorting techniques. Alternatively, the above infected cells are characterized by apoptosis and can thus be diagnosed on the known characteristics of apoptosis.

10 The invention furthermore provides or describes all steps needed for the construction of a recombinant, replication-defective adenovirus expressing the apoptosis-inducing agent apoptin. High titres of recombinant-apoptin adenovirus can be produced by means of

15 adenovirus packaging cell lines, such as 293, 911 and PER.C6. Apoptin does not exhibit a detectable negative effect on all necessary adenovirus replication steps and other adenovirus life-cycle events under cell culture conditions.

20 In addition, the invention describes the construction of a control recombinant adenovirus, which contains all sequences as the recombinant-apoptin adenovirus, but due to the 3'-5' orientation of the apoptin-encoding sequence under control of the regulating promoter elements, not able

25 to express apoptin. By means of this control adenovirus vector, the specific effects of apoptin expression by a recombinant adenovirus can be examined.

Recombinant replicative-defective adenovirus expresses apoptin in high amounts in various tumor and/or transformed

30 cells resulting in the induction of apoptosis. In contrast, expression of apoptin in normal non-transformed human cells by means of recombinant adenoviruses does not result in the induction of apoptin-induced apoptosis.

In particular, the invention relates to anti-tumor

35 therapies. Treatment of tumor(cell)s will take place by expression of apoptin by means of infecting (tumor)cells with gene delivery vehicles such as adenovirus vectors that

contain a coding sequence for a protein with apoptin-like activity. Therefore, the invention provides gene delivery vehicles such as the adenovirus expressing apoptin which is a very potential anti-tumor agent. Adenovirus regulation of

5   apoptin does not or at least not detectable induce apoptosis in normal cells, indicating that the toxicity of in-vivo treatment with recombinant-apoptin adenovirus will be low. By means of recombinant-apoptin adenovirus infection a much higher amount of apoptin-expressing

10 (tumor) cells can be achieved. This finding is an major improvement of apoptin expression in comparison to DNA transfections.

    The invention relates also to the construction of a VP2 expression unit without the synthesis of apoptin and/or

15 a part of apoptin. Furthermore, we have provided evidence that expression of the chicken anemia virus (CAV) protein VP2 enhances the apoptin-induced apoptosis in human tumor cells. To be more precise VP2 and apoptin acts synergistically concerning induction of apoptosis in tumor

20 cells. This finding indicates that co-expression of VP2 and apoptin will result in an improvement of apoptin-based therapies.

    The invention describes the significant improvement of apoptin expression by changing its direct upstream

25 sequences of the ATG-initiation codon. The improvement of expression does not need a amino acid change in the apoptin protein, as was predicted by the KOZAK rule. Improvement of upstream sequences of the ATG-initiation codon of the other CAV proteins will also result in improvement of their

30 synthesis.

    The invention also relates to the construction of retroviral vectors, which express apoptin in human tumor cells resulting in the induction of apoptosis. This result with recombinant-apoptin retrovirus in combination with the

35 recombinant-apoptin adenovirus data indicate that apoptin expression is not toxic for the replication of a DNA- and RNA-virus.

Expression of apoptin in (tumor) cells may also take place by infecting cells with other DNA and/or RNA-viral vectors, besides adenovirus or retrovirus vectors, that contain a coding sequence for apoptin. In addition,

5   virus-derived vector systems, such as plasmoviruses can be used for the induction of apoptin-induced apoptosis in tumor cells.

The invention will be explained in more detail on the

10 basis of the following experimental part. This is only for the purpose of illustration and should not be interpreted as a limitation of the scope of protection.

EXPERIMENTAL PART

15

Cells and cell culture conditions.

Ad5 E1-transformed human embryonic kidney (HEK; 293) and human embryonic retina (HER; 911 and PER.C6) cell lines were grown in Dulbecco's modified Eagle medium (DMEM)

20 supplemented with 10% fetal calf serum (FCS) in a 5% CO_2 atmosphere at 37oC. Cell line 293 was obtained from the American Type Culture Collection (ATCC CRL 1573). Cell lines 911 and PER.C6 were obtained from Fallaux et al. (1996). Cell culture media, reagents, and sera were

25 purchased from GIBCO Laboratories (Grand Island, NY). Culture plastics were purchased from Greiner (Nürtingen, Germany).

Human epidermal keratinocytes were isolated from foreskin and grown in the presence of a layer of mouse 3T3

30 fibroblasts lethally irradiated with 137-Cs. Primary cultures of keratinocytes (FSK-1) were initiated in complete medium as described (Rheinwald and Green, 1975) with minor modifications.

Tumorigenic keratinocytes, SCC-15 cells (Rheinwald and

35 Beckett, 1981), derived from squamous-cell carcinoma, were cultured in DMEM/F12 (3:1) medium containing 5% fetal calf serum, 0.4 ug hydrocortisone and 1 uM isoproterenol. The

human hepatoma-derived HepG2 cells (Aden et al., 1979) and the human osteosarcoma-derived U2OS and Saos-2 cells (Diller et al., 1990) were grown in DMEM (GIBCO/BRL) supplemented with 10% fetal calf serum. The spontaneously

5 transformed keratinocyte strain HaCaT (Boukamp et al., 1988) was a gift from Prof. Dr. R. Fusenig, DKFZ, Heidelberg, Germany. HaCAT cells were grown in DMEM supplemented with 10% fetal calf serum.

Murine cell lines were cultivated in Dulbecco's

10 modified Eagle medium with high glucose (4.5 gram per liter) and 10% fetal calf serum in a 5% CO_2 atmosphere at 37oC. The ecotropic packaging cell line Psi-2 (Mann et al., 1983) and the amphotropic packaging cell line PA317 have been described before (Miller, 1990a,b).

15

Virus techniques.

Plaque assays were performed as described previously (Fallaux et al., 1996).Briefly, adenovirus stocks were serially diluted in 2 ml DMEM containing 2% horse serum and

20 added to near-confluent 911 cells in 6-well plates. After 2h incubation at 37oC, the medium was replaced by F-15 minimum essential medium (MEM) containing 0.85% agarose (Sigma, USA), 20 mM HEPES (pH 7.4), 12.3 mM $MgCl_2$, 0.0025% L-glutamine, and 2% horse serum (heat-inactivated at 560C

25 for 30 minutes).

Small-scale production of adenovirus lots was performed as described previously (Fallaux et al., 1996). Briefly, near-confluent 911 ot PER.C6 monolayers were infected with approximately 5 plaque-forming units

30 (p.f.u._s) per cell, in phosphate-buffered saline (PBS) containing 1% horse serum. After 1 hour at room temperature, the inoculum was replaced by fresh medium (DMEM/2% horse serum). After 48 hours, the nearly completely detached cells were harvested, and collected in

35 1 ml PBS/1% horse serum. Virus was isolated from the producer cells by 3 cycles of flash-freeze/thawing. The

lysates were cleared by centrifugation at 3000 rpm fr 10 minutes, and stored at -20oC.

The 911 and PER.C6 produced rAdV stocks were screened for the presence of recombinant-competent adenovirus by
5   performing PCR analysis with primers derived from the Ad5 ITR region (5_-GGGTGGAGTTTGTGACGTG-3_) and the E1A encoding region (5_-TCGTGAAGGGTAGGTGGTTC-3_) as described by Noteborn and De Boer (1995) using a Perkin Elmer PCR apparatus. The presence of a 600-bp amplified fragment
10 indicates that replication-competent (E1-region containing) adenovirus exists in the analysed virus stock (Hoeben, unpublished results) or by infecting HepG2 cells with rAdV batch. During a period of at least 10 days, the cells were monitored for potential cytopathogenic effects and by
15 indirect immunofluorescence using a specific monoclonal antiserum directed against E1A protein.

<u>Plasmids and DNA transfections.</u>

The adaptor plasmid pMad5 was constructed from pMLP10
20 (Levrerno et al. 1991)as described below. Plasmid pMLP-10-lin was derived from pMLP10 by insertion of a synthetic DNA fragment with unique sites for the restriction endonucleases MluI, SplI, SnaBI, SpI, AsuII, and MunI into the HindIII site of pMLP10. The adenovirus
25 BglII fragment spanning nt 3328 to 8914 of the Ad5 genome was inserted into the MunI site of pMLP-lin. From the resulting plasmid, the SalI-BamHI fragment was deleted to inactivate the tetracycline resistance gene. The resulting plasmid was controlled by restriction-enxyme analysis and
30 named pMad5. Expression of genes inserted in the multiple cloning site will be driven by the adenovirus major late promoter, which in this configuration is linked to the adenovirus immediate-early gene 1 (E1) enhancer.

All CAV DNA sequences are originally derived from the
35 plasmid pIc-20H/CAV-EcoRI (Noteborn and De Boer, 1990). The expression plasmid pCMV-fs, formerly called pCMV-VP3

(Noteborn et al. 1994), contains CAV DNA sequences encoding apoptin exclusively (nt 427-868).

The plasmid pCMV-VP2mu (Noteborn, unpublished results) contains CAV DNA sequences of positions 380 to 1512. This CAV DNA fragment contains the coding region for VP2 flanked by 25 bp 5'-non-coding and 484 bp 3'-non-coding CAV DNA sequences. 106 bp downstream of the start codon for VP2 the start codon for apoptin is situated in another reading frame. To prevent the synthesis of apoptin without interfering the VP2 synthesis a mutation in the apoptin-initiation codon (ATG was changed into ACG) was introduced and in addition a point-mutation at position 549 (T was changed into an A), resulting in an extra stopcodon within the gene encoding apoptin. Therefore, the inserted CAV sequences will only express full-length VP2 protein. By indirect immunofluorescence was shown that VP2 can be produced but that apoptin was not synthesized.

For the cloning of PCR-amplified DNA fragments, we have used plasmid pCR-3.1, which was purchased commercially from InVitrogen (Carlsbad, CA). For the construction of a recombinant-apoptin replication-defective retrovirus, the retrovirusvector pLXSN was used (Miller, 1990a,b).

All cloning steps with plasmid DNAs were in principle carried out according the methods by Maniatis et al. (1992).

All used enzymes were commercially obtained from Boehringer Mannheim, Germany and/or BioLabs, USA.

Plasmid DNA was purified by centrifugation in a CsCl gradient and column chromatography in Sephacryl S500 (Pharmacia, Uppsala, Sweden). The human cell lines HaCAT, HepG2, SCC-15, 293, 911, and PER.C6 were transfected with plasmid DNA by calcium-phosphate precipitation as described by Graham and Van der Eb (1973). Normal human diploid keratinocytes (FSK-1; second passage), U2OS and Saos-2 cells were transfected with DOTAP (Fischer et al., 1996).

Indirect immunofluorescence assay.

Cells were fixed with 80% acetone and used for immunofluorescence assays with CAV-specific or adenovirus E1A-specific monoclonal antibodies and goat anti-mouse

5   and/or goat anti-rabbit IgC conjugated with fluorescein(Jackson Immunoresearch Laboratories Inc., West Grove PA), as described by Noteborn et al. (1990). Nuclear DNA was stained with 2,4-diamino-2-phenylindole (DAPI) or propidium iodide (PI).

10

RESULTS AND DISCUSSION

Construction of the adaptor vector pMab

To introduce a BamHI restriction-enzyme site into the

15   adaptor plasmid pMAd5, it was digested with the restriction enzyme ClaI and treated with calf intestine alkaline phosphatase. A ClaI-BamHI linker was treated with T4-kinase and ligated to itself by using T4-DNA ligase and subsequently by ClaI digestion. The ClaI/BamHI/ClaI linker

20   was isolated and ligated to the linearized pMad5 vector. The bacterial strain JM109 was transformed with the ligation products.

By restriction-enzyme digestions, the final vector pMab was characterized. By means of the pMab vector foreign

25   genes can be ligated into the unique BamH1 site under regulation of the adenovirus major late promoter. A schematic representation of pMad5 and pMab is shown in Figure 1.

30   Construction of a recombinant-apoptin and control adaptor vector

To construct a adaptor vector for introducing the apoptin gene into a adenovirus, pMab was treated with BamHI and subsequently with calf intestine phosphatase.

35   Subsequently, pCMV-fs was treated with BamH1 and a 0.45 kb DNA fragment containing the apoptin-encoding sequences was isolated. The apoptin DNA fragment was ligated into the

BamHI site of the linearized pMab adaptor vector. The
ligation products were cloned into the baterial strain
JM109. The orientation of apoptin in pMab was determined by
restriction-enzyme analysis.

5   The pMab construct containing the apoptin gene in the
5'-3' orientation under the regulation of the adenovirus
major late promoter will express the apoptin gene. This
adaptor vector is called pMab-VP3 and will be used to
generate a adenovirus vector expressing apoptin. The pMab
10   DNA plasmid containing the apoptin-encoding sequences in
the 3'-5' orientation downstream of the adenovirus major
late promoter cannot express apoptin and will be used to
make a control recombinant adenovirus vector. A schematic
representation of both recombinant adaptor vectors is shown
15   in Figure 2.

Induction of apoptosis by a CMV-plasmid versus a
recombinant-apoptin adaptor vector expressing apoptin.
       Firstly, we have examined whether the pMab-VP3 DNA
20   vector is indeed able to express apoptin in transfected
cells, whereas pMab-con should not do so. To that end,
human adenovirus-transformed 293 and 911 cells were
transfected with pMab-VP3, pMab-con, and as positive
control with pCMV-VP3. Approximately two days after
25   transfection, the cells were fixed and examined for
expression of apoptin by means of an
·indirect-immunofluorescence assay. The cell cultures
transfected with pCMV-VP3 and pMab-VP3 contained about 1%
of the cells reacting with an apoptin-specific monoclonal
30   antibody, whereas cell-cultures transfected with pMab-con
DNA did not. These results imply that pMab-VP3 expresses
apoptin and as expected pMab-con not.
       In an other transfection experiment, we have analysed
the induction of apoptosis in 911 cells after transfection
35   with pMab-VP3 versus pCMV-VP3. Three days after
transfection, the 911 cells were harvested and examined by
indirect-immunofluorescence for expression of apoptin. In

addition, the cells were stained with DAPI or PI, which stain intact DNA strongly, but apoptotic DNA weakly and/or irregularly (Telford, 1992).

Approximately 60% of the apoptin-positive 911 cells,
5   transfected with pMab-VP3 were apoptotic, whereas around 40% of the apoptin-postive cells, transfected with pCMV-VP3 underwent apoptosis. These results indicate that pMab-VP3-regulated expression of apoptin results in a similar or somewhat higher level of apoptosis induction,
10   than apoptin expressed by pCMV-VP3. The results are shown in Figure 3.

Furthermore, apoptin is able to induce apoptosis in human adenovirus-transformed cells, E1B does not inhibit apoptosis induced by apoptin. In contrast, E1B is able to
15   block apoptosis induced by a great variety of chemotherapeutic agents. These results indicate that apoptin is a very potent anti-tumor agent.

Construction of recombinant-apoptin adenovirus

20   Recombinant-apoptin adenovirus vectors were generated by co-transfection into helper cell line 911 of adaptor plasmids pMab-VP3 carrying the coding sequences for apoptin plus some adenovirus sequences, and plasmid DNA JM17 containing the entire adenovirus DNA minus the E1 and E3
25   region (McGrory et al., 1988). The co-transfections were transformed with calcium-phosphate-precipitated DNA as described by Graham and Van der Eb (1973). The recombinant adenovirus DNA is formed by homologous recombination between the homologous viral sequences that are present in
30   the plasmid pMAb-VP3 and the adenovirus DNA of JM17 DNA.

In a similar way, co-transfections of 911 cells were carried out with pMab-con and pJM17 DNA to generate the control recombinant adenovirus that cannot express apoptin, and which will be used as adenovirus control for the
35   apoptin-induced apoptotic effects.

Several hours after transfection, the 911 cell monolayers were covered with an agarose overlayer and

incubated at 37oC until recombinant adenovirus-induced plaques became clearly visible. The virus was harvested from plaques as PBS-horse serum stocks, as described by Fallaux et al. (1996). Subsequently, a part of the

5 recombinant-virus stocks was added to 24-wells containing fresh 911 cells. Several days later, these infected 911 cells were lysed and the recombinant viruses were harvested.

Next, the expression of apoptin by the potential

10 recombinant-apoptin adenoviruses (rAd-VP3) or its absence of expression by control recombinant adenoviruses (rAd-con) was examined. A part of the recombinant virus stocks derived from the infected 24-wells plates were used to infect fresh 911 cells, which were grown as monolayers on

15 glass cover slips. One day later, the infected 911 cells were fixed with aceton and analysed by immunofluorescence using the apoptin-specific monoclonal antibody 85.1. Five out of 5 analysed 911 cell cultures infected with putative rAd-VP3, contained cells expressing apoptin. None of the

20 911 cells infected with Ad-con and non-infected 911 cells were positive for apoptin.

These results imply that upon co-transfection of adenovirus packaging cell lines, such as 911 cells, with the required adaptor and adenovirus DNA, viable rAd-VP3

25 expressing apoptin can be generated.

Two stocks derived from rAd-VP3 or rAd-con plaques were used for purifying the rAds by carrying out three subsequent plaque purifications with 911 cells or in parallel a limited-dilution assay on PER.C6 cells as

30 described by Fallaux (1996).

Production of rAd-VP3 and rAd-con by using PER.C6 cells
Small-scale production of rAd-VP3 and rAd-con lots using PER.C6 cells were performed as described (Fallaux,

35 1996). Briefly, the procedure is described in the Experimental section.

By plaque-assay, the titres were determined to be approximately 10^{11-12} per ml cleared lysate for both rAd-VP3 and rAd-con. The obtained titres are not lower than observed in our laboratory for other rAd's.

5   By means of the PCR-analysis and infection of HepG2 with rAd-VP3 and rAd-con was examined whether the produced virus batches contained replication competent adenovirus (see also Experimental section). Both the rAd-VP3 and rAd-con batches were free of RCA, as proven by both

10   methods.

We conclude that the expression of apoptin does not negatively interfere with all required steps of the adenovirus life cycle under cell-culture conditions. Therefore, a gene-therapy based on an adenovirus vector

15   expressing apoptin is feasible.

Induction of apoptosis in human transformed and/or malignant cell lines

We have examined whether infection of human tumor

20   cells with rAd-VP3 will result in apoptin-induced apoptosis. To that end, human hepatoma HepG2, osteosarcoma U2OS cells, SCC-15 cells, derived from a squamous cell carcinoma, and cells from the spontaneously transformed keratinocyte cell line HaCaT were infected with rAd-VP3.

25   One day after transfection, the cells were fixed and by means of immunofluorescence and DAPI staining the cells were examined for apoptin synthesis, and whether they have underwent apoptosis. Already, 1 day after infection almost all analysed apoptin-positive human tumor cells were

30   apoptotic. In non-infected cultures only a few percent of the human tumor cells were apoptotic. The results for HepG2 and U2OS cells are shown in Figure 4.

These results indicate that rAd-VP3-expressed apoptin can induce apoptosis in different mammalian tumorigenic

35 . and/or transformed cell lines.

Expression of apoptin in normal cells infected with rAd-VP3

To analyse the effect of apoptin expressed by rAd-VP3 in infected normal non-transformed cells, FSK-1 cells were infected with rAd-VP3. Four days after transfection, the
5 cells were analysed by indirect immunofluorescence using the monoclonal antibody 85.1 and DAPI-staining. At most 8% of the apoptin-positive cells showed a DAPI-abnormal staining, indicating that they might have underwent (apoptin)-induced apoptosis. However, 7% of the cells that
10 were not infected also had a aberrant DAPI-stained DNA pattern. The results are shown in Figure 4.

These results indicate that rAd-VP3-directed expression of apoptin does not result in apoptin-induced apoptosis in normal non-transformed human cells, in
15 contrast to transformed/tumorigenic human cells.

Increasement of the synthesis of apoptin

To examine the effect of the direct sequences in front of the apoptin ATG-initiation codon, we have made two
20 pCR-3.1-apoptin constructs. pCR-VP3ori contains the original direct upstream sequences (5_-TTTCAA-3_) of the ATG-codon, whereas the other one, pCR-Vp3mu contains the direct upstream sequence 5_-GCCAAC-3_. By means of an in-vitro transcription/translation wheat-germ assay, it was
25 determined that the apoptin expression of pCR-VP3mu was at least 5 times more than observed for pCR-VP3ori. These data indicate that the nature of the direct upstream sequences of the apoptin-ATG influences the synthesis of apoptin. Construction of (viral) vectors with the direct upstream
30 sequence 5_-GCCAAC-3_ in front of the ATG-codon of apoptin, will result in a higher apoptin production and indirectly in an increased apoptin-induced apoptosis.

Important to mention is also that the amino-acid sequence of apoptin is not altered as predicted to be
35 necessary for increased translation efficiencies according the "Kozak rule" (Caventer and Stuart, 1991). According to this rule, we should have changed the nucleotide at

position +4 from an A into a G, resulting into a different second amino acid of apoptin which would have changed its activity.

5 Co-expression of VP2 and apoptin in human tumor cells synergistically increases the induction of apoptosis

To examine the effect of co-expression of VP2 and apoptin on the induction of apoptosis, Saos-2 cells were (co)-transfected with pCMV-fs, expressing apoptin and/or
10 pCMV-VP2mu, expressing VP2. The cells were fixed with aceton at various time intervals after transfection. By indirect immunofluorescence, the VP2-positive cells were determined with monoclonal antibody CVI-CAV-111.1 (Noteborn and Koch, 1996) and the apoptin-positive cells
15 with monoclonal antibody CVI-CAV-85.1. At day 3 after transfection, only 3% of the VP2-expressing cells underwent apoptosis, and only about 10% of the apoptin-expressing cells. In contrast, about 40% of the Saos-2 cells expressing both VP2 and apoptin were already apoptotic.
20 Also 4 days after transfection, the percentage of VP2/apoptin-positive cells that underwent apoptosis was significantly higher than in cells expressing apoptin or VP2 alone.

These results show that VP2 enhances the
25 apoptin-induced apoptosis and are shown in Figure 5.

Construction and production of rAD-VP2

To construct a recombinant Adenovirus expressing the viral protein 2 (VP2) of chicken anemia virus, the adaptor
30 plasmid pMAb-VP2 was made. A 1.1-kb BamHI fragment was isolated from the plasmid pCMV-VP2mu containing all VP2 coding sequences, but with 2 point mutations within the apoptin-coding region (see Experimental section) and ligated into the BamH1-linearized and calf intestine
35 alkaline phosphatase-treated adaptor vector pMAb. The final construct pMab-VP2 was characterized by restriction-enzyme and sequence analysis and shown in Figure 6.

By co-transfection of 911 cells with pMab-VP2 DNA and pJM17 DNA, rAD-VP2 was made. The co-transfection and all other required steps needed for characterization, purification and production of rAd-VP2, were carried out as
5   described for rAd-VP3 and rAd-con. By indirect immunofluorescence using monoclonal antibody CVI-CAV-111.1, it was shown that 911 and PER.C6 cells infected with rAd-VP2, indeed could express VP2 protein.

10   <u>Construction of a retrovirus vector expressing apoptin</u>

To generate plasmid pL-VP3-SN (see Figure 7), a BamHI fragment carrying the apoptin-coding sequences were inserted in the unique BamHI site of pLXSN. With restriction-enzyme analysis the proper orientation of the
15   insert was confirmed. To test the integrity of the insert the plasmid pL-VP3-SN was transfected with the calcium phosphate co-precipitation technique in COS-7 and HepG2 cells. Four days after transfection, the cells were fixed and analyzed with monoclonal antibody 85.1 for the
20   expression of the apoptin protein. In appoximately 1-2% of the cells, apoptin expression could be detected. The majority of the cells underwent apoptosis, as determined by DAPI staining. These data show that the proviral LTR promoter is capable of driving the expression of the
25   apoptin protein, that its gene is intact in the DNA construct and that in transfected HepG2 and COS-7 cells the expression of apoptin induces apoptosis.

To generate viruses the plasmid pL-VP3-SN was transfected into Psi-2 cells and into PA 317 cells with the
30   calcium phosphate co-precipitation technique. Fourty-eight hours after transfection, the supernatant of the cells wass harvested and dilutions were used to infect HepG2 cells (the PA317 supernatant) and NIH3T3 cells (the Psi-2 supernatant) in the presence of 4 ug/ml polybrene. Four
35   days after infection, the cells were fixed and analysed for apoptin expression by staining with the monoclonal antibody 85.1. Approximately 1% of the cells were found to express

apoptin. The majority of the apoptin-positive HepG2 cells were apoptotic. These data demonstrate that the cells had been transduced with the L-apoptin-SN retroviruses. In addition, it demonstrates that a single copy of the

5   provirus is sufficient to express sufficient amounts of the apoptin protein to be detected by immunofluorescence, and this amount is sufficient to induce apoptosis in a human tumor cell line, namely the hepatoma cell line HepG2.

Taken together, these data demonstrate that retrovirus
10   vectors carrying the apoptin gene can be generated and can be used to induce apoptosis in human tumor cells. It formally proves that neither the apoptin gene nor its expression interfere with essential steps in the retrovirus life cycle. It also demonstrates that apoptin-containing
15   retroviruses can be produced batch-wise in quantities sufficient to be used to transduce human tumor cells in tissue culture.

Diagnostic assay for cancer cells based on rAD-VP3
20   The cellular location of apoptin is different in tumorigenic/transformed human cells in comparison to normal non-transformed cells. Furthermore, another marker is the specific ability of apoptin to induce apoptosis in tumorigenic/transformed cells and not in normal cells.

25   By infecting cells with rAd-VP3 and analyzing the apoptin location and/or induction of apoptosis within these cells, one is able to prove whether a cell is malignant or not. Primary cells are isolated from (suspicious) tissue and cultured in the required medium. The cells are infected
30   with rAd-VP3 and in parallel with rAd-con, and subsequently analyzed. For instance, by using an immunofluorescence assay based on monoclonal antibodies specific for apoptin, 85.1. The cells will be checked for having apoptin in the cytoplasm (normal cells) or in the nucleus (transformed
35. cells). In addition or instead of, the percentage of apoptotic cells will be estimated. If the percentage of apoptotic cells is significantly higher for rAd-Vp3- than

for rAd-con-infected cells, these cells have become malignant.

DESCRIPTION OF THE FIGURES

Figure 1 shows the diagrammatic representation of the essential parts of the adenovirus adaptor vectors pMAd5 and pMab.

Figure 2 shows the diagrammatic representation of the
5   essential parts of the recombinant adenovirus adaptor vectors pMab-VP3 and pMab-con.

Figure 3 shows the apoptin-induced apoptosis activity in 911 cells transfected with pMAb-VP3 or pCMV-VP3. Two independently cloned and purified pMab-vP3 DNA-batches
10   (pMab-VP3/m1 and pMab-VP3-m2) were used for the transfection of 911 cells. The cells were fixed 3 days after transfection and analysed by indirect immunofluorescence using the apoptin-specific monoclonal antibody CVI-CAV-85.1 (85.1; Noteborn et al., 1991). The
15   percentage of cells that stained abnormally with DAPI is given as a relative measure for apoptosis.

Figure 4 shows the apoptin (called VP3) -induced activity of human tumorigenic hepatoma HepG2 cells, osteosarcoma U2OS cells and normal non-transformed diploid
20   FSK-1 keratinocytes infected with the recombinant-apoptin replication-defective adenovirus Ad-VP3. The cells were analysed by indirect immunofluorescence using the monoclonal antibody 85.1 and stained by DAPI. The HepG2 and U2OS cells were fixed 1 day after transfecion and the FSK-1
25   cells were harvested and fixed 4 days after transfection. The percentage of apoptin-positive cells that stained abnormally with DAPI is given as a measure for apoptin-induced apoptosis (black boxes). As control, the percentage of non-infected cells that have become
30   DAPI-abnormally stained is given (open boxes).

Figure 5 shows the apoptin- and/or VP2-induced apoptosis activity in Saos-2 cells transfected with 2.5 ug

pCMV-fs DNA expressing apoptin (formerly called pCMV-VP3; apoptin is named VP3) and 2.5 ug pCMV-neoBam DNA (Danen-Van Oorschot, 1997); or with 2,5 ug pCMV-VP2 DNA expressing the CAV protein 2 (VP2), and 2.5 ug pCMV-neoBam DNA; or with

5   2.5 ug pCMV-fs and 2.5 ug pCMV-VP2 resulting in the expression of both apoptin (VP3) and VP2. The cells were fixed 3, 4 and 5 days after transfection and analysed by indirect immunofluorescence using the apoptin-specific monoclonal antibody CVI-CAV-85.1 (85.1; Noteborn et al.,

10   1991)or with monoclonal antibody CVI-CAV-111.1 (Noteborn and Koch, 1996). The percentage of cells that stained abnormally with DAPI is given as a relative measure for apoptosis.

Figure 6 shows the diagrammatic representation of the

15   essential parts of the recombinant adenovirus adaptor vectors pMab-VP2.

Figure 7 shows the diagrammatic representation of the essential parts of the recombinant retrovirus transfervector pLS-VP3-N.

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protein derived from chicken anemia virus, induces a
p53-independent apoptosis in human osteosarcoma cells. Cancer
5   Research 55, 486-489.

CLAIMS

1. A gene delivery vehicle comprising a nucleic acid molecule encoding apoptin-like activity.

2. A gene delivery vehicle according to claim 1 additionally comprising a modified translation initiation site directly
5   upstream the ATG-initiation codon of said nucleic acid molecule.

3. A gene delivery vehicle according to claim 2 wherein said translation initiation site comprises the nucleic acid sequence GCCAAC.

10   4. A gene delivery vehicle comprising a nucleic acid molecule encoding VP2-like activity.

5. A gene delivery vehicle according to claim 4 additionally comprising a modified translation initiation site directly upstream the ATG-initiation codon of said nucleic acid
15   molecule.

6. A gene delivery vehicle according to any of claims 1 to 3 additionally comprising a nucleic acid molecule encoding VP2-like activity.

7. A gene delivery vehicle according to claim 6 additionally
20   comprising a modified translation initiation site directly upstream the ATG-initiation codon of the nucleic acid molecule encoding VP2-like activity.

8. A gene delivery vehicle according to any of claims 1 to 7 which is a virus.

25   9. A gene delivery vehicle according to claim 8 which is a replication-defective virus.

10. A gene delivery vehicle according to claim 8 or 9 which is an adenovirus.

11. A gene delivery vehicle according to claim 8 or 9 which
30   is a retrovirus.

12. A gene delivery vehicle according to any of claims 1 to 11 which additionally comprises at least one target molecule

13. A gene delivery vehicle according to claim 12 wherein the target molecule is reactive with a tumor cell surface receptor.

14. A host cell comprising a gene delivery vehicle according to any of the claims 1-13.

15. A host cell according to claim 14 which is a helper or packaging cell.

16. A host cell according to claim 14 which is selected from the group of HEK;293, HER;911, PER.C6, Psi-2 and PA317 cells.

17. Use of a gene delivery vehicle according to any of claims 1-13 in cancer treatment.

18. Use of a gene delivery vehicle according to claim 17 in combination with conventional (chemo)therapy.

19. Use of a gene delivery vehicle according to any of claims 1-13 for the treatment of hyperplasia, metaplasia and dysplasia.

20. Use of a gene delivery vehicle according to any of claims 1-13 in diagnosis.

21. Use of a gene delivery vehicle according to claim 20 in the *in vitro* detection of transformed, or cancerous, or hyperplastic, metaplastic or dysplastic cells.

ABSTRACT

The invention relates to gene delivery vehicles which comprise nucleic acid molecules encoding apoptosis-inducing proteins VP2 and/or apoptin (VP3) like activity. VP2 and VP3 are viral proteins of the Chicken Anaemia Virus.

Also, the invention relates to anti-tumor therapies. Infection of various human tumor cells with the gene delivery vehicles of the invention will result in the induction of apoptosis in tumor cells and much reduced apoptosis, if at all, in normal diploid, non-transformed/non-malignant cells.

Also the invention relates to the diagnosis of cancer, and related forms of hyperplasia, metaplasia and dysplasia.

FIGURE 1

pMad5 9270 bp
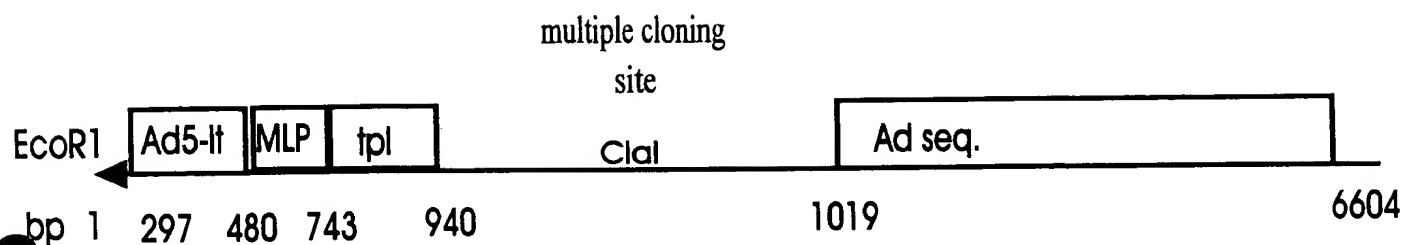
Ad5-5'PvuII (ITR, packaging signal)
Major late promotor (MLP)
Tripartite leader (tpl)
Ad5-BglII fragment (nt 3328-8914 Ad5)
b lactamase

BamH1-ClaI Linker
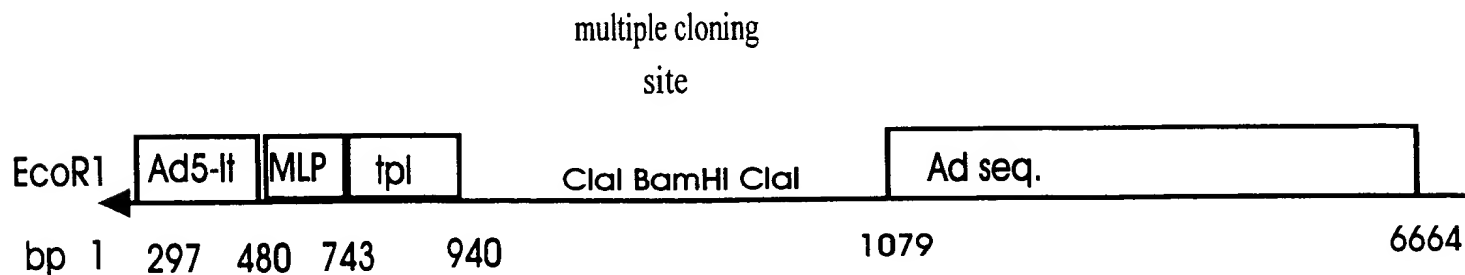
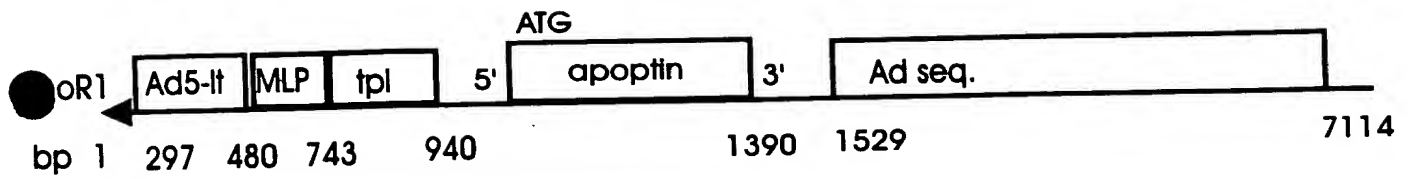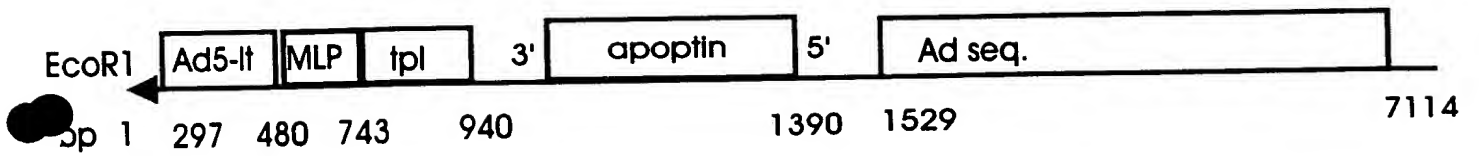5'-GATCCCGCACGCGTGCGAT-3'
3'- GGCGTGCGCACGCTAGC-5'

pMab   9306 bp

FIGURE 2

pMabvp3 9756 bp


```
                                    ATG
         ┌──────┬────┬─────┐    ┌──────────────┬────┬─────────────────────────────┐
●oR1     │Ad5-lt│MLP │ tpl │ 5' │   apoptin    │ 3' │   Ad seq.                   │
◄────────┴──────┴────┴─────┘    └──────────────┴────┴─────────────────────────────┘
bp  1   297   480   743      940                    1390  1529                      7114
```

pMabcon 9756 bp


```
         ┌──────┬────┬─────┐    ┌──────────────┬────┬─────────────────────────────┐
EcoR1    │Ad5-lt│MLP │ tpl │ 3' │   apoptin    │ 5' │   Ad seq.                   │
●◄───────┴──────┴────┴─────┘    └──────────────┴────┴─────────────────────────────┘
bp  1   297   480   743      940                    1390  1529                      7114
```

FIGURE 3

Ad transfer vector vs cmv vector

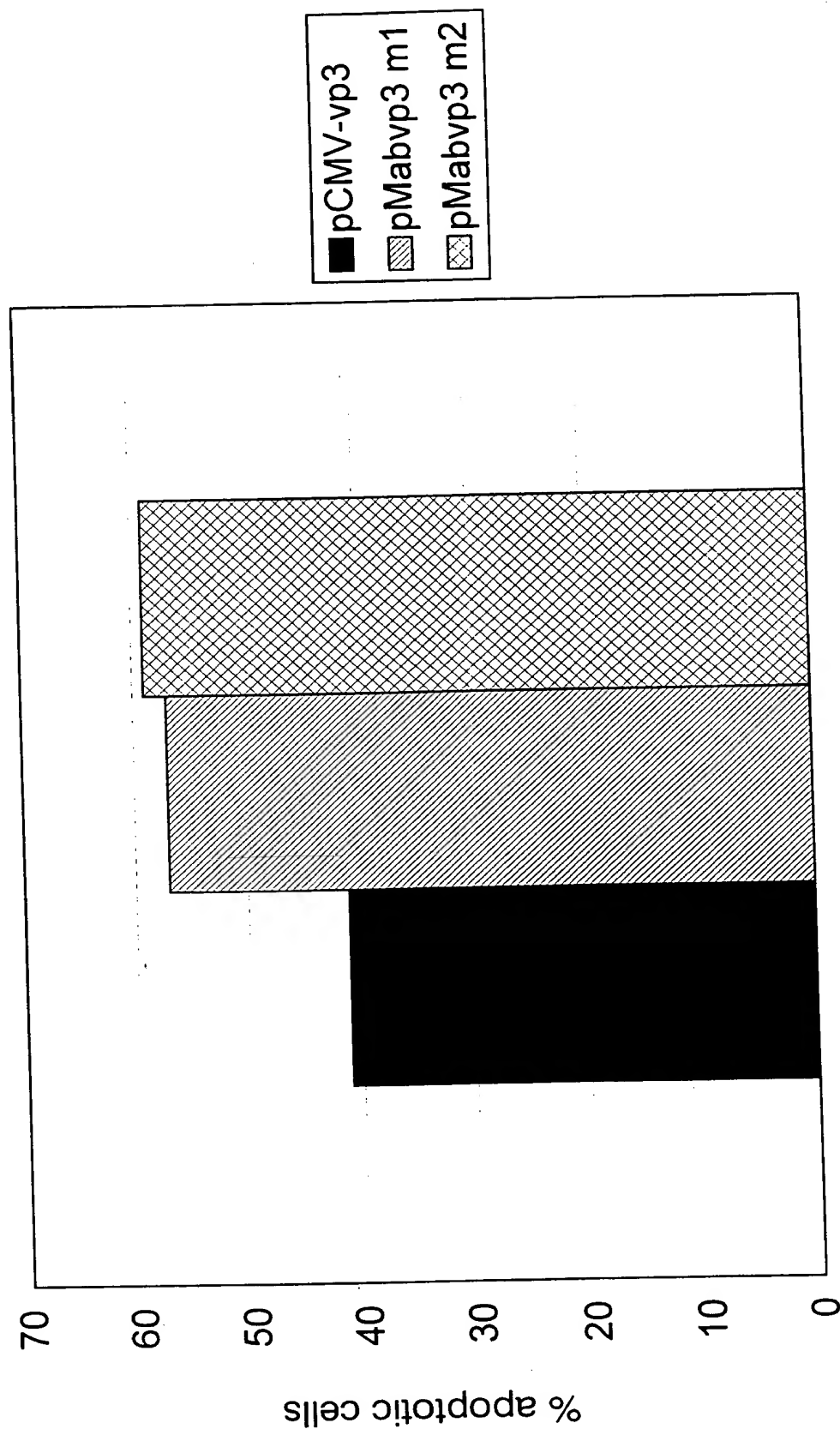## 911 3 days after transfection

- pCMV-vp3
- pMabvp3 m1
- pMabvp3 m2

% apoptotic cells

FIGURE 4

effect Ad-vp3 on transformed and normal cells

FIGURE 5

Influences of VP2 on VP3 induced apoptosis in saos-2 cells

n=2

☐ day 3
▨ day 4
■ day 5

corrected for desmin
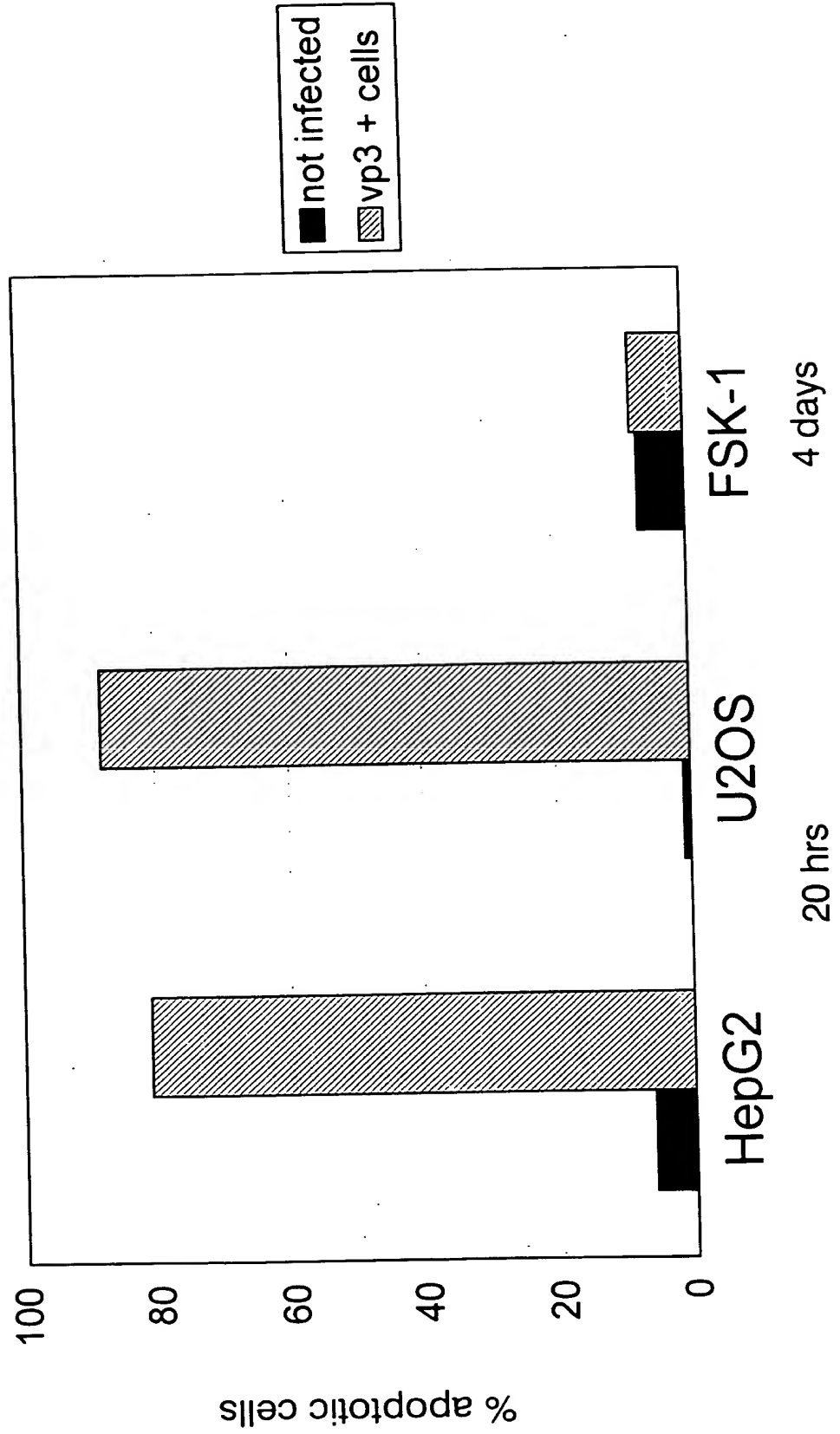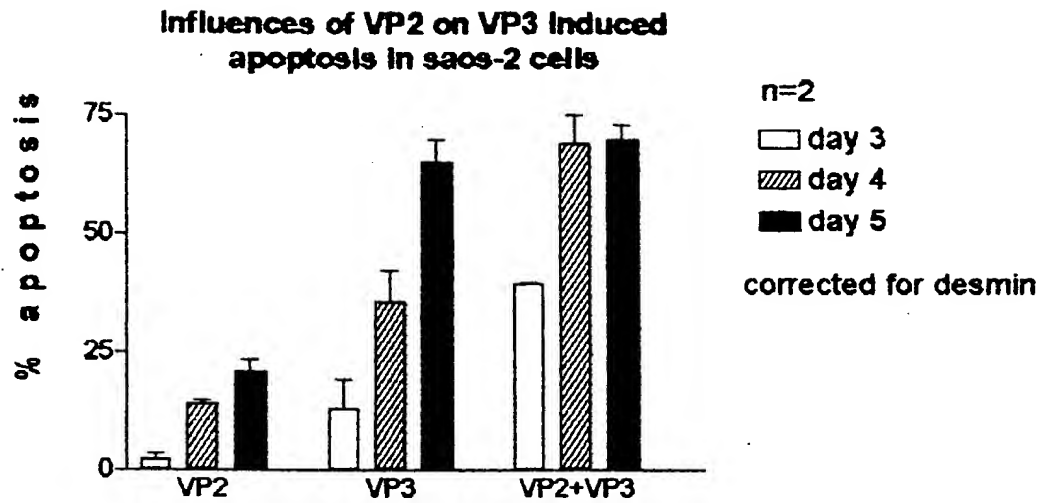
FIGURE 6
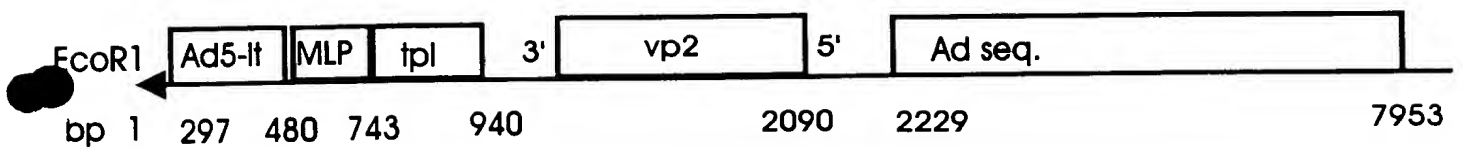
pMabvp2 10456 bp

pMabcon2 10456 bp

FIGURE 7

retroviral VP3 vector: